Characterization of Cells by Flow Cytometry

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Hematopoietic cells are traditionally identified by analysis of proteins expressed on the cell surface. These surface antigens are used as markers to characterize cell types, their stages of differentiation/maturation, and activation status. For example, B cells can be distinguished from other cell types by the expression of B220, an isoform of the membrane phosphatase CD45. However, B220 may also be expressed on other cell types, such as NK cells and some activated T cells. Therefore, more accurate characterization of B cells requires simultaneous staining of multiple surface markers. The analysis described uses specific antibodies coupled to one of four fluorochromes: fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), peridinin chlorophyll-a (PcP), and allophycocyanin (APC). These fluorochromes can be used simultaneously to stain and analyze the expression patterns of four different proteins in the same sample. The fluorochrome stained cell populations are analyzed using a FACSCalibur dual-laser flow cytometer.

Notes

Multiple combinations of surface markers are used to characterize each cell sample. The number of different combinations of fluorescent antibodies (or stains) to be used in characterizing the population determines the total cell number of experimental samples for each preparation. Use 5×10^5 to 1×10^6 cells per sample for each set of up to four color fluorochrome-coupled antibodies and for each control. If cell numbers are limiting, the minimum number of cells that can be stained and analyzed is approximately 5×10^4 per sample.

Positive and negative antibody staining samples must be included as controls to set up the flow cytometer. Each experiment should contain isotype-matched irrelevant antibodies (i.e., antibodies that do not recognize a specific antigen binding site on the cells), to determine the signal contributed by nonspecific antibody binding. Anti-keyhole limpet hemocyanin (KLH) antibodies are routinely used for this purpose. Unstained cells can be run to determine levels of autofluorescence alone. When staining for multiple markers with a mix of fluorochromes, each fluorochrome must be run individually (with substituted isotype matched antibodies for the other staining combination fluorochromes). If using a number of different antibodies coupled to the same fluorochrome in different samples, use the strongest staining antibody as the single positive control for that fluorochrome. Samples stained with the single fluorochromes are used to minimize the detection of specific fluorochromes with overlapping emission spectra within the channels defined by the optical filters for each fluorochrome. These compensation settings are initially set using the FACScomp program with the Calbrite beads, but are adjusted during set up for sample acquisition using the CellQuest program with appropriately stained mouse splenocytes.

Staining Procedure

1. Determine the number of samples (combinations of stains, controls, etc.) required for the analysis of each preparation of cells.
2. Transfer the total number of required cells for each experimental preparation to a microfuge tube (5×10^5 to 1×10^6 cells per stain/sample).
3. Add magnetic cell sorting buffer (MACS buffer) to bring the total volume of suspended cells to approximately 1 ml.
4. Centrifuge the cells at approximately 400 x g for 5 min at 4 °C. Aspirate the supernatant.
5. Resuspend the pelleted cells in MACS buffer containing 1 µg/ml of anti-CD16/32 (leukocyte Fc receptor [FcR] block recommended use ≤1 µg/million cells). Place on ice for 5 to 15 min. The amount of MACS buffer containing FcR block that is required equals 90 µl x the number of samples. Note: this antibody binds a common epitope in both CD16 (FcγRIII) and CD32 (FcγRII). As an alternative to using affinity purified MoAb, the 2.4G2 secreting cell line may be obtained from American Type Culture Collection (ATCC). Supernatant containing secreted antibody may be collected as hybridoma supernatant (MoAb concentration range for hybridoma supernatants is approximately 0.1 to 10 µg antibody/ml).
6. Aliquot 90-µl portions of each batch of FcR blocked cells into individual wells of a 96-well V-bottom microtiter plate. (Alternatively, if a small number of samples are being processed, cells can be added directly into the FACS tubes.)
7. Add 10 µl of the antibody mix containing the combination of fluorochrome-conjugated antibodies to the appropriate 90 µl of cells. (Dilute specific antibody mixes to 10 x the final specific antibody concentration in MACS buffer with 1 µg/ml of FcR block added.)
8. Seal the plate with adhesive strips and vortex to mix cells with antibodies. (If the samples are in the FACS tubes, stroke the tubes along a rack to mix.)
9. Stain the cells with fluorochrome-conjugated antibodies on ice, protected from light, for 30 min.
10. Centrifuge the microtiter plates at approximately 400 x g for 5 min at 4 °C. Pull off the adhesive tape and flick the plate over the sink to remove the supernatant. (If cells are stained in the FACS tubes, add 1.9 ml of MACS buffer to provide a wash, then centrifuge the tubes at 400 x g for 5 min at 4 °C.)
11. Add 100 µl of MACS buffer to each well of the V-bottom plate, seal the plate with adhesive, and then vortex to mix and wash the cells. (For cells washed in FACS tubes, pour off the supernatant, then add 500 µl of MACS buffer. These cells are now ready for FACS analysis.)
12. Centrifuge the V-bottom plates at 400 x g for 5 min at 4 °C. Pull off the adhesive tape and flick the plate over the sink to remove the supernatant.
13. Repeat steps 11 and 12 an additional 3 times.
14. Add 100 µl of MACS buffer and store cells on ice, protected from light, until ready to collect. If cells need to be stored overnight or for several days prior to collection by the FACSCalibur, add MACS buffer plus 1% paraformaldehyde in phosphate buffered saline (1% PFA solution) or Cytofix, and cells can be stored for 2 to 3 days prior to reading on the flow cytometer.

15. Transfer the 100 μl from each well of the V-bottom plate to separate FACS tubes containing 400 μL of MACS buffer (or Cytofix, where appropriate). Analyze using the FACSCalibur flow cytometer.

Reagents and Materials

Magnetic cell sorting buffer (MACS buffer): AfCS Solution Protocol ID PS0000000100

CD16/CD32 (Fcγ III/II Receptor) NA/LE: BD Pharmingen; catalog no. 553140

2.4G2 secreting cell line: American Type Culture Collection (ATCC); catalog no. HB-197

Microtiter plates, 96-well, V-bottom: Fisher Scientific; catalog no. 07-200-108

FACS tubes (Falcon polystyrene round-bottom tubes, 12 x 75 mm): Falcon; catalog no. 35-2054

Adhesive sealing film: Midwest Scientific; catalog no. 100SPS

Fluorescence-activated cell sorting buffer (FACS buffer): AfCS Solution Protocol ID PS0000002900

FACSCalibur flow cytometer: Becton Dickinson; catalog no. 343023

Cytofix: BD Pharmingen; catalog no. 2014KZ

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